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Toward Antibody-directed Enzyme Prodrug Therapy with the T268G Mutant of Human Carboxypeptidase A1 and Novel *in Vivo* Stable Prodrugs of Methotrexate*

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Antibody-directed enzyme prodrug therapy (ADEPT) has the potential of greatly enhancing antitumor selectivity of cancer therapy by synthesizing chemotherapeutic agents selectively at tumor sites. This therapy is based upon targeting a prodrug-activating enzyme to a tumor by attaching the enzyme to a tumor-selective antibody and dosing the enzyme-antibody conjugate systemically. After the enzyme-antibody conjugate is localized to the tumor, the prodrug is then also dosed systemically, and the previously targeted enzyme converts it to the active drug selectively at the tumor. Unfortunately, most enzymes capable of this specific, tumor site generation of drugs are foreign to the human body and as such are expected to raise an immune response when injected, which will limit their repeated administration. We reasoned that with the power of crystallography, molecular modeling and site-directed mutagenesis, this problem could be addressed through the development of a human enzyme that is capable of catalyzing a reaction that is otherwise not carried out in the human body. This would then allow use of prodrugs that are otherwise stable *in vivo* but that are substrates for a tumor-targeted mutant human enzyme. We report here the first test of this concept using the human enzyme carboxypeptidase A1 (hCPA1) and prodrugs of methotrexate (MTX). Based upon a computer model of the human enzyme built from the well known crystal structure of bovine carboxypeptidase A, we have designed and synthesized novel bulky phenylalanine- and tyrosine-based prodrugs of MTX that are metabolically stable *in vivo* and are not substrates for wild type human carboxypeptidases A. Two of these analogs are MTX-α-3-cyclobutylphenylalanine and MTX-α-3-cyclopentyltyrosine. Also based upon the computer model, we have designed and produced a mutant of human carboxypeptidase A1, changed at position 268 from the wild type threonine to a glycine (hCPA1-T268G). This novel enzyme is capable of using the *in vivo* stable prodrugs, which are not substrates for the wild type hCPA1, as efficiently as the wild type hCPA1 uses its best substrates (*i.e.* MTX-α-phenylalanine). Thus, the k_{cat}/K_m value for the wild type hCPA1 with MTX-α-phenylalanine is 0.44 μM^{-1} s^{-1}, and k_{cat}/K_m values for hCPA1-T268G with MTX-α-3-cyclobutylphenylalanine and MTX-α-3-cyclopentyltyrosine are 1.8 and 0.16 μM^{-1} s^{-1}, respectively. The cytotoxic efficiency of hCPA1–268G was tested in an *in vitro* ADEPT model. For this experiment, hCPA1-T268G was chemically conjugated to ING-1, an antibody that binds to the tumor antigen Ep-Cam, or to Campath-1H, an antibody that binds to the T and B cell antigen CDw52. These conjugates were then incubated with HT-29 human colon adenocarcinoma cells (which express Ep-Cam but not the Campath 1H antigen) followed by incubation of the cells with the *in vivo* stable prodrugs. The results showed that the targeted ING-1:hCPA1-T268G conjugate produced excellent activation of the MTX prodrugs to kill HT-29 cells as efficiently as MTX itself. By contrast, the enzyme-Campath 1H conjugate was without effect. These data strongly support the feasibility of ADEPT using a mutated human enzyme with a single amino acid change.

A current major challenge to cancer therapy is to increase antitumor selectivity. One approach to realizing this goal is to use the exquisite selectivity of the antibody:antigen reaction to target therapeutic entities specifically to tumors. While absolutely tumor-specific antibodies are not known, many antibodies are available that can deliver tumor-selective targeting (for review, see Ref. 1).

One investigational therapy that makes use of this principle of antibody targeting is antibody-directed enzyme prodrug therapy (ADEPT).[1] ADEPT is a powerful strategy with the potential for tumor-specific long-term delivery of chemotherapy (2–7). The premise of ADEPT is to target an enzyme of interest specifically to tumor cells by coupling it to a tumor-specific antibody. This conjugate is delivered to the patient systemically and then allowed to bind to the antigen-expressing target cells. Unbound conjugate is allowed to clear from circulation, and when the circulating levels of conjugate are sufficiently low, a prodrug is administered, also systemically, that can be converted to a toxic chemotherapeutic drug by the targeted enzyme-antibody conjugate. The action of the enzyme-antibody conjugate on the prodrug then ideally generates lethal levels of drug specifically at the tumor site. For this therapy to be

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Parts of this work have been reported in the patent literature (40) and in abstract form (41, 42).

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[1] The abbreviations used are: ADEPT, antibody-directed enzyme prodrug therapy; CPA, carboxypeptidase; hCPA, human CPA; bCPA, bovine CPA; MTX, methotrexate; HPLC, high pressure liquid chromatography; WT, wild type; Sulfo-SMCC, sulfosuccinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate.

lective, however, nonspecific activation of the prodrug at es distant to the tumor must be minimized. This is generally complished by using a conjugate enzyme with an activity not ıdogenous to the host or at least not accessible to the prodrug -29).

A number of ADEPT strategies have been reported (2–29). he concept has been shown to be effective both in *in vitro* and ı *vivo* models, and at least one ADEPT strategy is currently ndergoing clinical evaluation (27–29). ADEPT *in vitro* efficacy as been demonstrated with enzyme-antibody conjugates of (*a*) arboxypeptidase G2 along with several nitrogen mustards 14–16), (*b*) alkaline phosphatase with phosphorylated prolrugs of mitomycin, a phenol mustard, and etoposide (3, 10, 11), (*c*) β-lactamase with lactam prodrugs of doxorubicin, vinca ılkaloid analogs, and a nitrogen mustard (7, 12, 13, 17, 18), (*d*) penicillin-G amidase with prodrugs of palytoxin, doxorubicin and melphalan (8, 9), (*e*) penicillin-V amidase with a prodrug of doxorubicin (2, 7), (*f*) human or *Escherichia coli* β-glucuronidase with glucuronide prodrugs of epirubicin, doxorubicin and a nitrogen mustard (22–24), (*g*) cytosine deaminase and 5-fluorocytosine (25, 26), and (*h*) bovine carboxypeptidase A and α-amino acid prodrugs of MTX (19–21). Further, *in vivo* antitumor efficacy has been shown in a number of systems using the enzymes alkaline phosphatase, carboxypeptidase G2, β-lactamase, and β-glucuronidase (2, 3, 7, 10–13, 15–18, 24).

An inherent problem with antibody-targeted therapies is the immune response mounted by the host to the foreign proteins and other antigens used in the therapy (1, 28). For example, monoclonal antibodies used in antibody targeting-based therapies are in general rodent in origin and as such recognized by the immune system (1, 28). ADEPT has the additional problem that the enzyme used to generate the site-specific drug synthesis can also be immunogenic, especially when a foreign enzyme is used. The immunogenicity associated with these foreign antibody or enzyme proteins decreases the utility of the antibody-targeting strategies by decreasing the ability of the physician to perform multiple dosing regimens.

Attempts are being made to overcome the immune response to the rodent antibodies through "humanization" of the antibodies (30). In this strategy, much of the sequence of the mouse monoclonal antibody is replaced with corresponding human antibody sequence. Only selected residues at the antigen combining site are left intact, leaving relatively few "rodent residues" remaining in the antibody.

We reasoned that the imunogenicity associated with the use of enzymes of nonhuman origin might be circumvented through a similar strategy. However, we chose not to precisely follow the strategy of antibody humanization, which commences the process with the binding site of a foreign protein. Rather, our approach to generate a composite human/nonhuman enzyme was to start with a fully human enzyme and change the "active site" at one or two residues to produce a >99.5% human enzyme capable of efficiently performing a non-human reaction. This human enzyme with non-human specificity, along with humanized antibodies, should then facilitate the production of enzyme:antibody conjugates having lower immunogenicity and benefit the development of multiple dosing regimen ADEPT strategies.

Our initial target to generate a human enzyme with non-human specificity was human pancreatic carboxypeptidase A, recently cloned and expressed in our laboratory (31). Pancreatic carboxypeptidase A is a zinc-containing exopeptidase released into the small intestine from the pancreas as a zymogen (28, 33). The CPA has two further subclasses, CPA1 similar and both enzymes prefer aromatic C-terminal amino acids, CPA2 enzyme prefers bulkier aromatic C-terminal amino acids (31, 37). This was shown for the rat enzyme with di- and tripeptide substrates and for the human with amino acid prodrugs of MTX (31, 37). High resolution crystal structures for bCPA have been determined (32). Of the nine active site residues within 4.5 Å of the bound substrate, only three vary among bovine CPA (32), rat CPA1 (36), rCPA2 (37), hCPA1 (31, 34), and hCPA2 (31, 35). These changes, at residues 253, 254, and 268, describe a larger binding pocket for CPA2 than CPA1 and provide a rationale for the substrate specificities.

Huennekens and co-workers (19–21) developed an *in vitro* ADEPT approach using MTX prodrugs in which MTX was modified at the α-carbon of the glutamate moiety with one of several natural amino acids. These prodrugs were relatively nontoxic in cell culture but could be activated by bovine CPA or carboxypeptidase B to form MTX. The most successful of these prodrugs was MTX-Phe, which was found to be an excellent substrate for bovine CPA (21). This system was effective under cell culture conditions where the IC_{50} of MTX-Phe against L1210 cells changed from 2.2×10^{-6} to 6.3×10^{-8} M, indistinguishable from MTX itself, in the presence of bovine CPA or a bovine CPA-antibody conjugate (21). An important positive aspect of this system is its use of MTX with its well known efficacy and toxicity profile (38, 39). Thus, MTX maximum tolerated doses are due to well understood gut and bone marrow toxicities. Therefore, specific generation of high concentrations of MTX at tumor sites distal from these sites of known toxicity should not have major side effects. Two potential limitations to the use of the bCPA/MTX-Phe system in humans, however, are the possible background activation of prodrug by endogenous hCPA to liberate MTX systemically and the immune response elicited by the bovine protein.

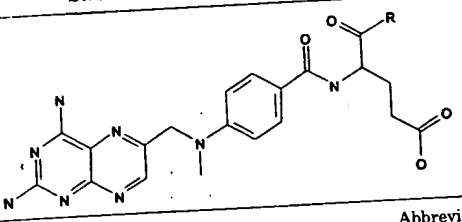
We sought to improve upon the MTX-Phe/bCPA system in two ways. First, we sought to use the human rather than bovine CPA. Second, we sought to change the catalytic specificity of the human enzyme(s) to accommodate MTX prodrugs that are not substrates for the endogenous wild type carboxypeptidases and would therefore be expected to be stable *in vivo*. The strategy chosen to accomplish this goal exploited parallel computer-aided design of novel active sites and of new MTX-α-amino acid prodrugs. Specifically, wild type hCPA and novel hCPA mutant active sites were designed using protein homology model building from the well known high resolution crystal structure of bCPA (40). Then these computer-generated active site mutants and similarly generated modified substrates were evaluated together and compared with wild type enzyme-substrate complexes. Favorable mutants and modified substrates were prepared and tested *in vitro* and *in vivo*. We report here MTX prodrugs that are stable *in vivo*, a one-amino acid mutant of hCPA1 that can efficiently use these *in vivo* stable prodrugs, and the use of these prodrugs along with a mutant hCPA enzyme-antibody conjugate for antigen-specific cytotoxicity *in vitro*. The work reported demonstrates proof of principle for the methodology for developing a very efficient mutant human enzyme/prodrug combination for use in ADEPT.

EXPERIMENTAL PROCEDURES

Materials

Human CPAs were obtained as described previously (31). Cell lines were obtained from ATCC (Rockville, MD) and grown in 90% RPMI 1640, 10% fetal calf serum at 37 °C under 5% CO_2. HT-29 cells for *in vitro* ADEPT experiments in human serum were taken from this medium and grown for 3 weeks in 95% RPMI 1640, 5% human serum at

TABLE I
Structures of MTX-α-R prodrugs



Compound	–R[a]	Abbreviation
GW1311	Phenylalanine	MTX-Phe
Negatively charged prodrugs		
GW2310	Glutamate	MTX-Glu
GW3347	Aspartate	MTX-Asp
GW3855	2-Carboxyphenylalanine	MTX-2-carboxy-Phe
GW3199	3-Carboxytyrosine	MTX-3-carboxy-Tyr
GW4694	3-Carboxyphenylalanine	MTX-3-carboxy-Phe
Bulky aromatic prodrugs (phenylalanine-based)		
GW4160	2-Iodophenylalanine	MTX-2-iodo-Phe
GW1667	1-Naphthylalanine	MTX-naphthyl-Ala
GW250	2-Cyclopentylphenylalanine	MTX-2-cyclopentyl-Phe
GW1442	2-Cyclohexylphenylalanine	MTX-2-cyclohexyl-Phe
GW3352	3-Cyclobutylphenylalanine	MTX-3-cyclobutyl-Phe
GW1834	3-*t*-Butylphenylalanine	MTX-3-*t*-butyl-Phe
GW637	3-Cyclopentylphenylalanine	MTX-3-cyclopentyl-Phe
GW827	3-(3-*n*-Pentyl)phenylalanine	MTX-3-*n*-pentyl-Phe
Bulky aromatic prodrugs (tyrosine-based)		
GW1867	3,5-Diiodotyrosine	MTX-3,5-diiodo-Tyr
GW2159	2-Cyclopentyltyrosine	MTX-2-cyclopentyl-Tyr
GW5798	3-Cyclobutyltyrosine	MTX-3-cyclobutyl-Tyr
GW3335	3-*t*-Butyltyrosine	MTX-3-*t*-butyl-Tyr
GW5755	3-Cyclopentyltyrosine	MTX-3-cyclopentyl-Tyr

[a] All R-groups are L-amino acids attached to the α-carboxyl of MTX via a standard peptide bond.

Methods

Synthesis of MTX Prodrugs

The synthesis of the MTX prodrugs shown in Table I is described elsewhere (40).

Mutagenesis of hCPA1

pMP36HCPA1 (31) containing pro-hCPA1-WT cDNA (as a fusion with yeast α factor leader, described below) was restricted with *Nco*I and *Sal*I to liberate a 481-base pair cDNA fragment. This fragment begins at nucleotide 893, proceeds to the 3′-end of the hCPA1 cDNA, and encodes amino acids 186–309 of mature hCPA1.[2] The CPA1 *Nco*I-*Sal*I fragment was ligated into the *Nco*I and *Sal*I cloning sites of pGEM5zf(−) (Promega) to generate pHCPAINS. pHCPAINS was restricted with *Sph*I and *Sal*I to liberate the hCPA1 *Nco*I-*Sal*I fragment, and an additional nine base pairs of pGEM5zf(−) sequence. This *Sph*I-*Sal*I fragment was cloned into M13mp19 (Life Technologies, Inc.) using its *Sph*I and *Sal*I cloning sites to generate M13mp19HCPA1.

Single-stranded M13mp19HCPA1 DNA was used as template for oligonucleotide-directed mutagenesis using the T7-GEN *in vitro* mutagenesis kit (U.S. Biochemical Corp.). The following mutagenic oligonucleotide primers (Oligos Etc.) were used to mutate residues Ile^{255} (AAT) and Thr^{268} (ACC) either separately or in tandem (mutagenic codons underlined): I255A, 5′-ggT CCA gTC AgC AgT gCT TCC-3′ (Ala = gCT); T268A, 5′-gAg CTC gAA ggC gAA ggA gTA-3′ (Ala = gCC); T268G, 5′-gAg CTC gAA gCC gAA ggA gTA-3′ (Gly = ggC). Using these oligonucleotide primers, the following hCPA1 mutants were formed: T268A, T268G, I255A, and I255A/T268G. Each of the mutagenized hCPA1 cassettes was sequenced to verify that only the desired DNA mutations were produced.

Expression of hCPA Enzymes in Yeast

Expression of hCPA enzymes in *Saccharomyces cerevisiae* was performed according to the strategy of Gardell *et al.* (43) as described by Laethem *et al.* (31). The cDNAs for pro-hCPA1 or pro-hCPA2 were cloned into the pMP36 vector and fused in frame to the yeast α factor leader of this vector using a polymerase chain reaction approach (44).

pMP36HCPA1 was restricted with *Nco*I to liberate a 1.2-kilobase pair fragment that was cloned into the *Nco*I site of the M13mp19HCPA1 mutants. The correct orientation of the *Nco*I fragment within M13mp19pro-hCPA1 mutants was verified, and the DNAs were restricted with *Hin*dIII and *Sal*I liberating a 1.2-kilobase pair cDNA encoding the entire pro-hCPA1 mutant enzyme. This fragment was ligated into the *Hin*dIII and *Sal*I sites of pMP36 yielding pMPHCPA1 mutants. These DNAs were restricted sequentially with *Bam*HI, *Sal*I, and *Ssp*I with intervening purifications by either phenol extractions or use of Promega Magic Mini Columns with manufacturer supplied procedures. Following *Ssp*I restriction, the 2.8-kilobase pair band, containing hCPA1 mutant with the yeast α factor leader in frame, was gel-purified from a 1% low melting agarose gel. The *Bam*HI-*Sal*I fragment was ligated into the pBS24.1 shuttle vector overnight at 16 °C. This vector (pBSHCPA1-mutant) was electroporated into DH5α, and plasmid DNA was isolated using the Wizard Miniprep Kit (Promega).

Approximately 500–2000 ng of pBSHCPA1 mutant DNA was electroporated into 40 μl of electrocompetent DLM101α *S. cerevisiae*. One ml of 1 M sorbitol was added immediately after electroporation to rescue the cells. 100-μl samples were plated out on dishes of yeast nitrogen broth-uracil selection medium and were incubated at 30 °C for 2–3 days (31). Positive colonies were picked and grown in yeast nitrogen broth-leucine selection medium containing 8% glucose at 30 °C for 48 h. This culture was used to seed 350 liters of YP, 1% glucose in a 500-liter New Brunswick fermenter.

Mutagenesis and Expression of hCPA2

Mutagenesis of hCPA2 was analogous to CPA1 described above. The following mutagenic oligonucleotide primer (Oligos Etc.) was used to mutate residue Ala^{268} (gCC): A268G, 5′-CAg TTC AAA gCC AAA TgA gTA-3′ (Gly = ggC). Using this oligonucleotide primer, the following hCPA2 mutant A268G was formed. The mutagenized hCPA2 cassette was sequenced to verify that only the desired DNA mutations were produced.

The hCPA2-A268G mutant was subcloned into pMP36, then into pBS, and expressed in yeast (described above).

Purification of Expressed hCPA1 and hCPA2 Enzymes

The wild type and mutant hCPA1 and hCPA2 enzymes were purified to electrophoretic homogeneity using a combination of hydrophobic and ion exchange chromatography as described previously (31).

Spectrophotometric Enzymatic Assays

Enzymatic activity was determined in one of two ways. Hippuryl-L-phenylalanine and hippuryl-DL-phenyllactate were determined spectrophotometrically at 255 nm as described (45). Reactions contained either 0.5 mM hippuryl-L-phenylalanine or 1.0 mM hippuryl-DL-phenyllactate in 25 mM Tris-HCl (pH 7.4), 100 mM NaCl. Reactions were initiated by the addition of enzyme and were monitored by the change in absorbance at 255 nm at 25 °C. Enzyme kinetic rates were determined from initial velocities using $\epsilon = 390\ M^{-1}$.

Hydrolysis of MTX prodrugs was measured using a modification of the coupled assay described by Kuefner *et al.* (19). Reactions were carried out in 1 ml of 25 mM Tris-HCl (pH 7.4), 100 mM NaCl at 25 °C. Buffer was added to the cuvette along with 0.026 units of carboxypeptidase G; then prodrug was added, and the absorbance was determined to calculate the concentration. Reactions were initiated by adding a known amount of CPA enzyme, and the decrease in absorbance at 255 nm was monitored. Enzyme kinetic rates were determined from initial velocities using $\epsilon = 9.57\ mM^{-1}$ for MTX. One unit of enzyme activity is defined as the hydrolysis of 1 μmol of substrate/min at 25 °C.

Thermal Stability Studies

Thermal inactivation of the enzymes was determined spectrophotometrically using the CPA assay described above. Inactivation reactions were carried out by incubating 0.1 mg/ml enzyme in Dulbecco's phosphate-buffered saline at the desired temperature. Aliquots were sampled at various times and assayed with hippuryl-DL-phenyllactate.

Stability of Prodrugs in Pancreatic Juice

This parameter was determined by incubating the prodrugs in sin activated human pancreatic juice. The percentage of conver MTX was determined as a function of time from linear co Pancreatic juice was a generous gift of Dr. T.

TABLE II
In vivo stability and biodistribution of MTX-Phe (GW1311) upon injection of 50 mg/kg to CD-1 nu/nu mice

Time	Tissue	Plasma	Liver	Kidney	Lg. int.	Sm. int./panc.[a]	Spleen
30 min	% recovered as MTX-Phe[b]	31	95	100	65	17	81
	MTX-phe level, nmol/g[b]	17.8	335	26.9	19.3	17.3	19.9
	MTX level, nmol/g[b]	39.6	18	0	10.4	84.5	4.7
120 min	% recovered as MTX-Phe[b]	3	8	0	22	0	0
	MTX-Phe level, nmol/g[b]	1.1	0.6	0	1.1	0	0
	MTX level, nmol/g[b]	35.7	7.1	0	3.9	203	1.4

[a] Small intestine and pancreas were analyzed as one tissue due to the difficulty of surgically resolving them.
[b] Each tissue was analyzed for MTX-phe and MTX. Percentage (%) recovered as MTX-phe = 100(MTX-Phe)/(MTX-Phe + MTX) for the particular tissue analyzed. Limit of detection = 0.5–1 nmol/g = 3 × background.

prodrugs. The pancreatic juice was activated fresh for each experiment by trypsinization with 1 mg/ml trypsin for 10 min at 37 °C. (This high concentration of trypsin was required to overcome endogenous trypsin inhibitors.) This activated solution was used directly for stability tests of the prodrugs. Activated pancreatic juice was diluted 1:40 to 1:2000 into 25 mM Tris-HCl, 100 mM NaCl, pH 7.5. Prodrug was then added to a final concentration of 50 μM. The solution was then incubated at 25 °C for up to 24 h. During this incubation period, aliquots were removed and analyzed by HPLC for prodrug and MTX. HPLC conditions were as follows. Chromatography was performed on a Waters C-18 Nova Pak column with a flow rate of 1 ml/min. Mobile phase conditions were a step gradient system composed of 0.1% trifluoroacetic acid in water (component A) or in acetonitrile (component B). For chromatography, the column was equilibrated with 82% A, 18% B. At 3 min, conditions were switched to 50:50 A:B. Then the column was reequilibrated starting at 7 min for another 10 min with 82:18 A:B. Under these conditions, MTX eluted at 4 min, and the prodrugs eluted at 6–8 min, depending upon hydrophobicity. Elution was monitored at 310 nm. No significant conversion of prodrug to MTX occurred when a suitable dilution of trypsin replaced activated pancreatic juice, indicating that conversion was due solely to materials contained in the pancreatic juice.

In Vivo Stability of Prodrugs

This parameter was measured as described elsewhere (42). Briefly, animals were dosed with prodrug intravenously. At specified times, plasma and tissues were collected and snap-frozen. The samples were then homogenized in 0.1 M HCl and extracted with a mix of 2 volumes of the HCl homogenate and 5 volumes of −20 °C acetonitrile. Then prodrug and MTX were measured by HPLC as described (42).

Conjugation of Mutant hCPA to Antibody ING-1 or Campath-1H

Enzyme Modification—The enzyme was modified at free amines with Sulfo-SMCC. This bifunctional reagent has both an amine-reactive *N*-hydroxysulfosuccinimide group and a thiol-reactive maleimide group. Since carboxypeptidase has no free thiols, the compound reacts with enzyme amines to place a maleimide on the enzyme for subsequent reaction/coupling with free thiols on the antibody.

Four mg of mutant or wild type carboxypeptidase A were combined with 0.15 mg of Sulfo-SMCC (Pierce) in 400 μl of Dulbecco's phosphate-buffered saline. The resulting solution was stirred for 45 min at 25 °C. The modified enzyme then resolved from reagent through a 1 × 13-cm G-25 medium column equilibrated with Dulbecco's phosphate-buffered saline.

Maleimide Content of the Activated Enzyme—Maleimide content was determined as follows. A 0.5-ml aliquot of a 6.3 μM solution of modified enzyme was mixed with 6 μl of 1 mM mercaptoethanolamine. The mixture was allowed to sit for 30 min to permit the enzyme-bound maleimide to react with the mercaptoethanolamine. Then 20 μl of 4 mg/ml Ellman's reagent was added to react with the remaining free mercaptoethanolamine. After another 20 min, absorbance at 412 nm was determined. The amount of enzyme-bound maleimide was inferred from the amount of mercaptoethanolamine that was consumed during reaction with the enzyme. Based upon a molar absorptivity of 13.6 mM^{-1}, the purified product was typically found to contain 1 maleimide/enzyme molecule. The derivatization had no effect upon enzyme activity measured with hippurylphenylalanine.

Antibody Modification—The antibody was modified with the amine-reactive reagent 2-iminothiolane (Traut's Reagent; Pierce), which rephate-buffered saline and 0.1 M triethanolamine-HCl, 2 mM EDTA, pH 8.0, under anaerobic conditions. This was allowed to react with stirring for 1 h and 45 min. The modified antibody was purified through a 1 × 13-cm G-25 medium column equilibrated with 0.02 M sodium acetate, 0.1 M NaCl, pH 5.8, bubbled with and maintained under a helium atmosphere.

Coupling of Antibody with Enzyme—The modified antibody was collected directly from the G-25 column into the solution of the modified carboxypeptidase A. The resulting mixture was then carefully adjusted to pH 7.4 with NaOH, made anaerobic by repeated N_2 gassing and evacuation, and allowed to react with stirring at 4 °C for 18 h. Excess free maleimide groups were removed by reacting the solution with 0.3 mM mercaptoethanolamine at room temperature for 1 h, and the solution was then concentrated to 1 ml. The resulting concentrated conjugate was purified from aggregates, unreacted enzyme, and small molecules by chromatography on Superose 12 HR 10/30.

Cell Culture ADEPT Experiments

IC_{50} values for drugs and prodrugs alone were determined as described previously (48). For ADEPT experiments, HT-29 cells were seeded at 7500 cells/well in 96-well plates containing 200 μl of 95% RPMI 1640/5% human serum (human growth medium). The cells were allowed to grow for 24 h at 37 °C. Then medium was removed, and 50 μl of conjugate composed of either ING-1-hCPA1-T268G or Campath-1H-hCPA1-T268G in fresh human growth medium was added to triplicate wells. Conjugate concentrations of 0, 2, 10, and 50 μg/ml were used. After 1 h at 37 °C in a 5% CO_2 incubator, conjugate was removed, and the plates were washed three times with fresh human growth medium without conjugate. Finally, prodrugs at 0, 0.01, 0.03, 0.1, 0.3, 1, 3, and 30 μM were added in 200 μl of fresh human growth medium, and cells were allowed to grow for 72 h. The plates were then stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium to assay for proliferation as described (48). MTX was included in separate wells in all experiments as an internal control for IC_{50} reproducibility.

RESULTS

Lack of in Vivo Stability of MTX-Phe—Vitols *et al.* (21) reported that MTX-Phe is a good substrate for bovine CPA. Only one pancreatic CPA isozyme has been found in this species; however, in rodents and humans two isozymes exist. We reported previously that the compound is a good substrate for both human isozymes, hCPA1 and hCPA2 (31). Thus, an hCPA-antibody conjugate should effectively hydrolyze MTX-Phe for human enzyme-based ADEPT as had been shown previously for a bovine CPA-antibody conjugate (21).

For ADEPT, it is desirable that the active agent (MTX) is generated selectively at the site of the tumor by the action of a targeted enzyme (CPA). Therefore, the prodrugs used must not be converted to the active agent systemically by the host. The *in vivo* conversion of MTX-Phe to MTX was tested in mice after intravenous administration. After administration of 50 mg/kg prodrug, animals were killed at 0.5 and 2 h, and tissues and blood plasma were collected and analyzed for both MTX-Phe and MTX. Table II shows the results of this experiment, where the data are presented as the percentage of sample found as

TABLE III
Comparison of rate of prodrug pancreatic juice hydrolysis with CPA enzyme kinetics

Compound	Pancreatic juice (% of MTX-Phe hydrolysis rate)	hCPA1[a]			hCPA2[a]		
		V_{max}	K_m	k_{cat}/K_m	V_{max}	K_m	k_{cat}/K_m
		μmol/min/mg	*mM*	*1/M(s)*	*μmol/min/mg*	*mM*	*1/M(s)*
MTX-Phe	100	3.24	0.0043	440,000	8.06	0.056	90,000
Negatively charged prodrugs							
MTX-Glu	0.12	0.094	0.1	170	0	ND[b]	0
MTX-Asp	0.06	0.063	0.5	80	0	ND	0
MTX-2-carboxy-Phe	0	0.003	0.7	3	<0.0005@50 μM	ND	ND
MTX-3-carboxy-Tyr	0.008	0.009	0.5	10	0.01	0.2	40
MTX-3-carboxy-Phe	0.27	0.08	30	1550	0.63	120	3100
Bulky aromatic prodrugs (phenylalanine based)							
MTX-2-iodo-Phe	ND	3.42	0.017	120,000	36	0.05	420,000
MTX-naphthyl-Ala	67	0.16	0.065	1400	36.9	0.016	1,400,000
MTX-2-cyclopentyl-Phe	0.17	0.021	0.06	200	0.11	0.081	770
MTX-2-cyclohexyl-Phe	0.0067	0	ND	0	0	ND	0
MTX-3-cyclobutyl-Phe	0.053	0	ND	0	0.086	0.2	260
MTX-3-*t*-butyl-Phe	0.02	0	ND	0	0.04	0.3	80
MTX-3-cyclopentyl-Phe	0.004	0	ND	0	0.012	0.3	25
MTX-3-*n*-pentyl-Phe	0	0	ND	0	0	ND	0
Bulky aromatic prodrugs (tyrosine-based)							
MTX-3,5-diiodo-Tyr	0.093	0	ND	0	0.31	0.3	610
MTX-2-cyclopentyl-Tyr	0.14	0.43	0.4	575	1	0.6	910
MTX-3-cyclobutyl-Tyr	0.0067	0	ND	0	0	ND	0
MTX-3-*t*-butyl-Tyr	0.0013	0	ND	0	0	ND	0
MTX-3-cyclopentyl-Tyr	0.002	0	ND	0	0	ND	0

[a] Limit of detection for V_o was 0.0006 μmol/min/mg for hCPA1 and 0.002 μmol/min/mg for hCPA2. Assuming a K_m of ~50 μM, the limit of k_{cat}/K_m detection = 5 and 20/(M)(s), respectively.
[b] ND, not determined.

of the largest accumulation of prodrug at 30 min (335 nmol/g, and 95% of the sample found in the liver was prodrug); however, by 2 h, MTX predominated in this tissue as well (0.6 nmol/g and 8% prodrug). The site of most rapid accumulation of MTX was the small intestine and pancreas, which was found to to have 84.5 nmol/g MTX and 17.3 nmol/g MTX-Phe at 30 min. By 2 h, MTX levels in this tissue were 203 nmol/g, and prodrug was not detectable. Overall the data show that MTX-Phe was rapidly converted to MTX in the mice, and significant amounts of the generated MTX were then observed in circulation. This rapid systemic generation of large amounts of MTX appeared to us to make MTX-Phe unsuitable for ADEPT.

In Vitro Stability of MTX-Phe in Human Pancreatic Juice—To overcome the *in vivo* metabolism problem, the source of MTX-Phe metabolism was investigated. *In vitro* tissue metabolism experiments showed that the compound was stable in plasma and only slowly metabolized in Ref. 42. We reasoned that other potential sources of this metabolism are the pancreatic CPAs that are secreted into the small intestine. This reasoning is consistent with the large MTX accumulation we observed in the small intestine and pancreas (Table II). These pancreatic enzymes enter the small intestine through the duodenum in the solution known as pancreatic juice. The material can be obtained from patients with pancreatic fistulae and used as a source of the human enzymes; indeed, human CPA was originally purified from this source (33). We used this material intact as a source of all pancreatic enzymes secreted into the small intestine to predict the stability of MTX-Phe in the small intestine. MTX-Phe was rapidly hydrolyzed to MTX by this material. A 1:100 dilution of pancreatic juice, hydrolyzed 50% of 50 μM MTX-Phe in 17 min at 25 °C. MTX-Phe is not a good substrate for trypsin, chymotrypsin, or carboxypeptidase B, and since it is a good substrate for both hCPA1 and hCPA2 (31), MTX-Phe. The comparison was then used as the primary test of new prodrugs designed and synthesized in the current program to be more stable *in vivo* (Table III). The results from the stability in pancreatic juice were then confirmed with subsequent *in vivo* experiments (42).

Design and Testing of MTX Prodrugs Predicted to Be Stable in Vivo—The active site model of hCPA1 was generated as described elsewhere (40) from the crystal structure of bovine CPA (32) using the Composer modeling program. This model is shown in Figs. 1 and 2*A*. The hCPA2 model is similar. The exceptions to this are a Gly in place of Ser at 253, Ser in place of Thr at 254, and an Ala in place of Thr at residue 268 (31). These substitutions participate to make a larger binding pocket in hCPA2 compared with hCPA1 and are presumably responsible for the hydrolysis of bulkier substrates by hCPA2 *versus* hCPA1 as has been reported for both the murine and human enzymes (31, 37).

The models of hCPA1 and hCPA2 were used to design MTX prodrugs that would not be substrates for either enzyme. Suitability of the designed and synthesized prodrugs for ADEPT was assessed by assaying them with WT hCPA1 and hCPA2 and testing their stability in pancreatic juice (and in mice; Ref. 42). Three general types of structural modification were explored for enhancement of prodrug stability. These included (*a*) the introduction of a negative charge to the prodrug amino acid moiety, (*b*) the addition of bulk to Phe- or Tyr-based prodrugs, and (*c*) the addition of both a charge and bulk.

Fig. 2 shows that the active sites of hCPA1 and hCPA2 are highly hydrophobic. Therefore, MTX-Glu, MTX-Asp, MTX-2-carboxy-Phe, MTX-3-carboxy-Phe, and MTX-3-carboxy-Tyr were produced. All of these compounds would introduce a negatively charged carboxyl group into the hydrophobic pocket and as such were not expected to be good hCPA substrates. The activities of these compounds with hCPAs and their relative

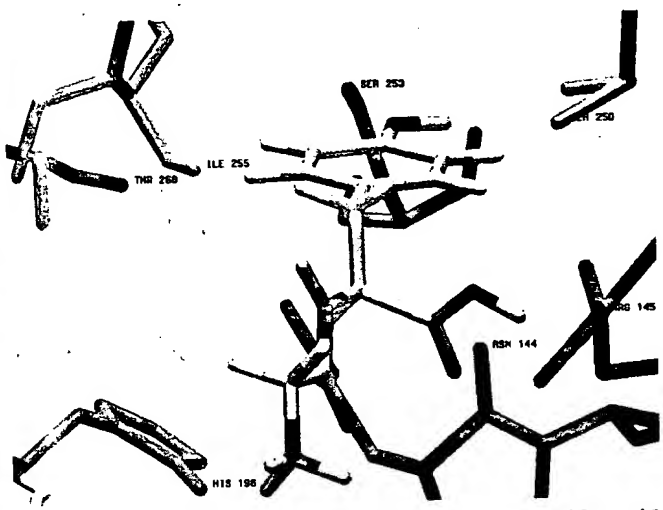


FIG. 1. **Representation of the human carboxypeptidase A1 binding pocket with slow substrate glycyltyrosine docked into the hydrophobic aromatic ring binding region.** The substrate is *color-coded* as follows. *Gray*, carbon atoms; *red*, oxygen atoms; *blue*, nitrogen atoms; *white*, hydrogen atoms. Residues close to the substrate binding pocket are displayed without hydrogens and *colored* to match the following scheme. *Yellow*, Ala, Ile, Leu, Val; *blue*, Arg, Lys; *purple*, Asn, Gln; *red*, Asp, Glu; *green*, Cys, Met, Ser, Thr; *white*, Gly, Pro; *light blue*, His; *gray*, Phe, Trp, Tyr.

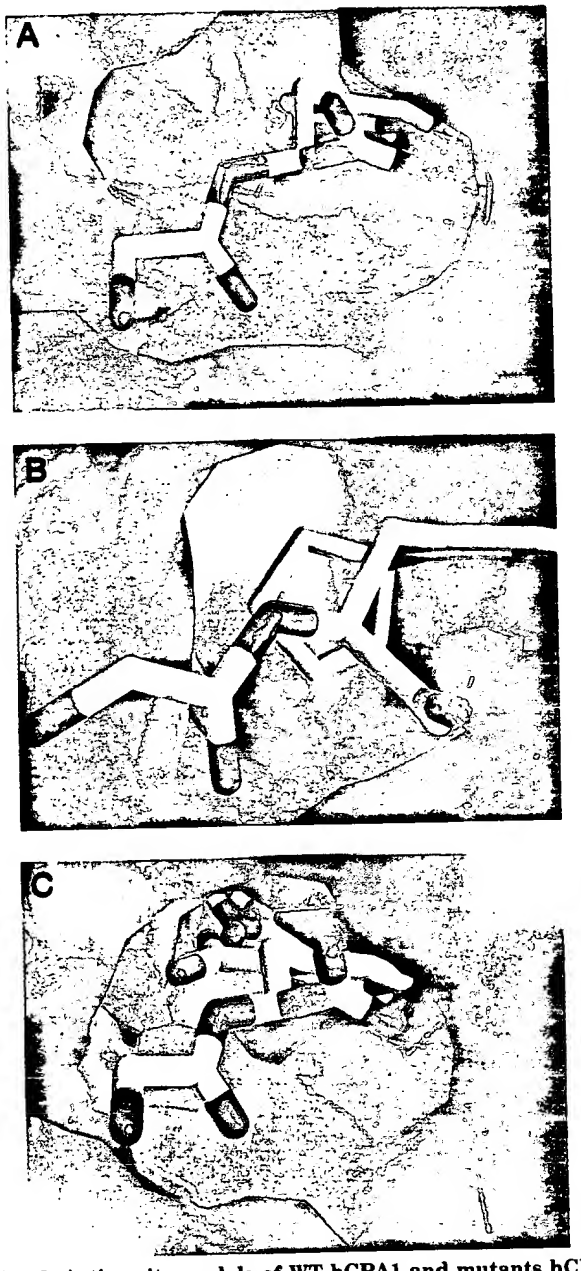


FIG. 2. **Active site models of WT hCPA1 and mutants hCPA1-T268G and hCPA1-I255H generated from the crystal structure of bovine CPA (32) using the Composer modeling program (40).** *Color coding* is the same as in Fig. 1; hydrogen atoms are not shown. *A*, color-coded surface model of hydrophobic binding pocket of wild type hCPA1 with glycyltyrosine docked and shown as a *stick figure*. *B*, *color-coded* surface model of the hydrophobic binding pocket of hCPA1-T268G with glycyl 3-cyclopentyltyrosine docked. The *white* region toward the cyclopentyl group is the T268G. *C*, *color-coded* surface model of hydrophobic binding pocket of hCPA1-I255H with glycyl 2-carboxytyrosine docked. The imidazole ring of I255H visible in the back of the pocket formed a salt bridge with the carboxyl group of the candidate substrate model.

MTX-Phe. The greatest pancreatic juice stability and poorest enzyme activity was found with the 2-carboxy-Phe and 3-carboxy-Tyr prodrugs, which introduce both bulk and a negative charge into the prodrug. Surprising, MTX-3-carboxy-Phe was a much better substrate than either MTX-2-carboxy-Phe or MTX-3-carboxy-Tyr and consequently far less stable in pancreatic juice. The reasons for this are not clear but may represent an ability of the 3-carboxy-Phe substituent to access the *para*-hydroxyl binding site of tyrosine-based substrates.

Fig. 2*A* shows that Thr^{268} of hCPA1 closely abuts the 2- or 3-hydrogen of the glycyl-Tyr slow substrate bound in this model. Ala^{268} of hCPA2 provides more space. This size restriction was used to design bulky prodrugs that were not expected to be either hCPA1 or hCPA2 substrates. This was accomplished by adding hydrocarbon substituents to the 2- or 3-positions of Phe or Tyr. The prodrugs produced, along with their enzyme activities and pancreatic juice stabilities are shown in Table III. Bulky substituents were very effective in decreasing the activity of the prodrugs toward hCPA1, hCPA2, and pancreatic juice hydrolysis. As predicted from our model and from previous work (31, 37), bulky prodrugs were in general poorer substrates for hCPA1 than hCPA2. Both Phe- and Tyr-based prodrugs were made with 2-cyclopentyl, 3-cyclobutyl, 3-*t*-butyl, and 3-cyclopentyl substituents. The data indicate that (when the activities were measurable, non-zero) k_{cat}/K_m for both hCPA1 and hCPA2 and relative pancreatic juice hydrolysis followed the order 2-cyclopentyl > 3-cyclobutyl > 3-*t*-butyl ≫ 3-cyclopentyl for both the Phe and Tyr prodrugs.

The activities of both hCPA1 and hCPA2 contributed to the pancreatic juice hydrolysis rate; therefore, pancreatic juice stability required that a prodrug be stable toward both hCPAs. This can be seen for example with MTX-Phe, MTX-naphthyl-Ala, MTX-2-cyclopentyl-Phe, MTX-3-cyclopentyl-Phe, and MTX-3-cyclopentyl-Tyr, which had decreasing total hCPA1 plus hCPA2 activity and increasing pancreatic juice stability. The k_{cat}/K_m values for MTX-Phe with hCPA1 and hCPA2, respectively, were 440,000 M^{-1} s^{-1} and 90,000 M^{-1} s^{-1}. The naphthyl-Ala being nearly as unstable in pancreatic juice as MTX-Phe. The k_{cat}/K_m for MTX-2-cyclopentyl-Phe with hCPA1 was 200 M^{-1} s^{-1}, and for hCPA2 it was 770 M^{-1} s^{-1}, which resulted in a pancreatic juice hydrolysis rate of 0.17% of MTX-Phe. The k_{cat}/K_m of MTX-3-cyclopentyl-Phe with hCPA1 was not measurable, and with hCPA2 it was 25 M^{-1} s^{-1}, resulting in a pancreatic juice hydrolysis rate of 0.004% of MTX-Phe.

TABLE IV
Thermal stability of WT and mutant hCPAs

Sample	$t_{1/2}$			
	4 °C	25 °C	37 °C	50°C
		h		
WT hCPA1	Long[a]	>170[b]	140	0.77
hCPA1-T268A	Long	>170[b]	>96[b]	0.93
hCPA1-T268G	Long	80	24	0.17
hCPA1-I255A	Long	8	1	
hCPA1-I255A/T268A	Long	8	1	
WT hCPA2	Long	>170[b]	>170[b]	8.75
hCPA2-A268G	Long	>170[b]	140	5.1

[a] At 4 °C, all the mutants were stable for at least 2 months. In addition, both WT enzymes, the 268G mutants, and the T268A mutant have been stored at this temperature for 2 years with little or no loss.
[b] Less than 10% decrease in activity of the enzyme over the stated time period.

TABLE V
Specific activity of WT and mutant hCPAs with hippuryl-Phe and hippuryl-Phe lactate

Assay conditions were as follows: 0.5 mM L-hippuryl-Phe or 1 mM DL-hippuryl-Phe lactate, at 25 °C in 25 mM Tris-HCl, 100 mM NaCl, pH 7.4.

Enzyme	Specific acitvity	
	Hippuryl-Phe	Hippuryl-Phe lactate
	μmol/min/mg	
WT hCPA1	7.2	790
hCPA1-T268A	7.4	160
hCPA1-T268G	20	60
hCPA1-I255A	8	350
hCPA1-I255A/T268A	12	50
WT hCPA2	8.7	2800
hCPA2-A268G	4.9	1900
Bovine CPA	24.1	310
Rat CPA1	11	560

MTX-Phe. As shown elsewhere (42), compounds with pancreatic juice hydrolysis rates in the range of 0.01% of MTX-Phe or less had excellent *in vivo* stability.

Design and Testing of Mutants of hCPA Predicted to Efficiently Hydrolyze Prodrugs That Are Stable in Vivo—Our next goal was to utilize our structural models of hCPA1 and hCPA2 to model mutant hCPAs into which the "stable prodrugs" could be docked. Fig. 2, *B* and *C*, shows models of two such mutants of hCPA1, I255H and T268G. The former was designed to introduce a positive charge into the active site to bind the carboxyl-containing prodrugs. The latter was designed to produce an active site that would bind Phe- or Tyr-based prodrugs substituted at the 2- or 3-position. I255K and I255H/T268G were also designed for the charged prodrugs, and T268A, I255A, and I255A/T268A were also designed to accept bulk. The A268G mutant of hCPA2 was also predicted and made for the bulky prodrugs. Expression and isolation of the hCPA1 mutants T268A, T268G, I255A, and T268A/I255A and the hCPA2 mutant A268G were accomplished using the yeast expression system described under "Experimental Procedures." All of these mutants were synthesized and secreted by the yeast as catalytically active enzymes. Unfortunately, no hCPA protein or enzymatic activity could be found in yeast expressing I255K, I255H, or I255H/T268G. Presumably, these three proteins misfolded and were degraded prior to secretion.

Enzyme Stability—Several of the WT and mutant enzymes were characterized for their stability in phosphate-buffered saline at 4, 25, 37, and 50 °C. The results from these experiments are shown in Table IV. All enzymes are quite stable at 4 °C. The I255A mutants were the least stable, since they exhibited half-lives of 8 h at 25 °C while all other mutants were stable for several days at this temperature. At 37 °C, WT hCPAs as well as hCPA1-T268A and hCPA2-A268G lost little activity after 2 days, and while hCPA1-T268G was less stable it also had a half-life of 24 h. At 50 °C, differential stabilities were clearer. At this temperature, the order of stability was WT hCPA2 > hCPA2-A268G > hCPA1-T268A > WT hCPA1 > hCPA1-T268G. In summary, these data show that the mutants of hCPA are active and stable.

Enzyme Kinetics—Specific activities of these enzymes with the model substrates hippuryl-Phe and hippuryl-Phe lactate are shown in Table V. As can be seen, all of these enzymes hydrolyzed these substrates efficiently. Interestingly, WT

(I255H, I255K, and I255H/T268G) could not be isolated. The hCPA mutants designed for the bulky hydrophobic prodrugs were not expected to efficiently hydrolyze charged prodrugs. However, mutation of hCPA1 to T268G produced a 5-fold enhancement in k_{cat}/K_m for the hydrolysis of MTX-3-carboxy-Phe, a 50-fold enhancement in the k_{cat}/K_m for hydrolysis of MTX-3-carboxy-Tyr and a 250-fold enhancement in the k_{cat}/K_m for hydrolysis of MTX-2-carboxy-Phe. The A268G mutation of hCPA2 was without effect on any of the charged prodrugs measured. The mutation of hCPA1 to T268A produced a modest enhancement in the hydrolysis of two of these prodrugs intermediate between the WT and T268G rates. These rate enhancements are likely due to the additional tolerance in the T268G and T268A active sites for bulk at the 2- and 3-positions of the substrate aromatic ring (as described below). The enhancements by hCPA1-T268G are probably inadequate for ADEPT, however, since the absolute k_{cat}/K_m values for these reactions were all quite small compared with that for hCPA1 with MTX-Phe (approximately 1000-fold less).

Bulky Aromatic Prodrugs—Table VII shows the activity of the hCPA mutants with the bulky hydrophobic prodrugs from Table I. The 268G mutants were designed to remove unfavorable interaction between the enzyme and 2- or 3-substituted MTX-Phe or MTX-Tyr, allowing their efficient binding and hydrolysis. As shown in Table VII, this goal was realized; the mutant hCPA1-T268G proved to efficiently hydrolyze a variety of substrates with bulky 2- or 3-position substituents ($k_{cat}/K_m \geq 100{,}000\ M^{-1}\ s^{-1}$). In contrast, the T268A mutant is a much poorer enzyme with all of these substrates. Thus, the minimal replacement of methyl with hydrogen at residue 268 produced dramatic rate enhancements. The hCPA2-A268G mutant also provided dramatic rate enhancements for some prodrugs; however, k_{cat}/K_m for the mutant with none of the stable prodrugs (pancreatic juice hydrolysis rate ≤0.01% of MTX-Phe) exhibited k_{cat}/K_m approaching $100{,}000\ M^{-1}\ s^{-1}$. Therefore, this enzyme was not as efficient as hCPA1-T268G with these compounds.

Since the active site of the WT hCPA2 has Ala at position 268, the activity of the hCPA1-T268A mutant was expected to parallel that of WT hCPA2. Data in Tables VI and VII demonstrate that this was observed, since the activity of hCPA1-T268A followed that of WT hCPA2 for most prodrugs assayed

TABLE VI
Kinetics of charged prodrugs with hCPA1-T268G, hCPA1-T268A, and hCPA2-A268G

Compound	hCPA1-T268G				hCPA1-T268A				hCPA2-A268G			
	V_{max}	K_m	k_{cat}/K_m	$(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}$	V_{max}	K_m	k_{cat}/K_m	$(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}$	V_{max}	K_m	k_{cat}/K_m	$(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}$
	μmol/min/mg	*mM*	*1/M(s)*		*μmol/min/mg*	*mM*	*1/M(s)*		*μmol/min/mg*	*mM*	*1/M(s)*	
MTX-Glu	0.012	0.06	120	0.7	ND[a]	ND	ND	ND	0	ND	ND	
MTX-ASP	0.024	0.25	60	0.75	ND	ND	ND	ND	0.12	0.9	76	
MTX-2-carboxy-Phe	0.1	0.09	670	250	0.007	0.06	60	20	<0.002@50 μM	ND		ND
MTX-3-carboxy-Tyr	0.06	0.07	500	50	0.01	0.1	60	6	0.02	0.2	70	2
MTX-3-carboxy-Phe	0.87	0.07	7200	4.6	0.11	0.05	1300	0.8	1.1	0.25	2500	0.8

[a] ND, not determined. Detection limits are as in Table III.

TABLE VII
Kinetics of bulky aromatic prodrugs with hCPA1-T268G, hCPA1-T268A, and hCPA2-A268G

Compound	hCPA1-T268G				hCPA1-T268A				hCPA2-A268G			
	V_{max}	K_m	k_{cat}/K_m	$(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}$	V_{max}	K_m	k_{cat}/K_m	$(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}$	V_{max}	K_m	k_{cat}/K_m	$(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{wt}$
	μmol/min/mg	*mM*	*1/M(s)*		*μmol/min/mg*	*mM*	*1/M(s)*		*μmol/min/mg*	*mM*	*1/M(s)*	
Phenylalanine-based compounds												
MTX-Phe	3.74	0.0003	7,350,000	17	3.46	0.0009	2,250,000	5.1	3.1	0.045	41,000	0.45
MTX-2-iodo-Phe	13.7	0.0004	*2.7E+07*	230	7.56	0.009	480,000	4.1	ND[a]	ND	ND	ND
MTX-naphthyl-Ala	1.15	0.0005	1,400,000	1,000	10.8	0.01	640,000	460	21.5	0.002	5,500,000	4
MTX-2-cyclopentyl-Phe	4.25	0.016	160,000	790	0.15	0.06	1,600	8	2.6	0.008	190,000	250
MTX-2-cyclohexyl-Phe	0.094	0.08	700	>100[b]	0.009	0.06	100	>5[b]	0.3	0.19	900	>50[b]
MTX-3-cyclobutyl-Phe	5.2	0.002	1,800,000	>400,000[b]	0.21	0.16	750	>40[b]	0.163	0.047	2100	8
MTX-3-*t*-butyl-Phe	1.3	0.002	380,000	>80,000[b]	0	ND	ND	ND	0.1	0.022	2700	34
MTX-3-cyclopentyl-Phe	2.35	0.015	92,000	>20,000[b]	0.002	0.03	30		0.02	0.013	1000	40
MTX-3-*n*-pentyl-Phe	0.18	0.008	14,000	>3,000[b]	0	ND	ND	ND	0	ND	ND	ND
Tyrosine-based compounds												
MTX-3,5-diiodo-Tyr	2.73	0.033	53,000	>10,000	ND	ND	ND	ND	2.7	0.0001	520,000	850
MTX-2-cyclopentyl-Tyr	1.82	0.026	41,000	70	ND	ND	ND	ND	3.6	0.008	260,000	280
MTX-3-cyclobutyl-Tyr	5	0.012	280,000	>60,000[b]	0.036	0.05	450	>20[b]	0.017	0.002	5200	>300[b]
MTX-3-*t*-butyl-Tyr	0.2	0.001	110,000	>20,000[b]	ND	ND	ND	ND	0.049	0.003	9000	>400[b]
MTX-3-cyclopentyl-Tyr	5.04	0.019	160,000	>30,000[b]	0	ND	ND	ND	0.023	0.002	7000	>400[b]

[a] ND = not determined. Detection limits are as in Table III.
[b] See Table III, footnote *a*.

of I255A/T268A was similar to that of T268A. This suggests that position 255 has little impact on substrate specificity in this class of aromatic substrates.

Overall the most useful mutant was hCPA1-T268G, since it was able to efficiently metabolize several of the bulky, stable MTX prodrugs. The most useful substrates for the enzyme, based upon large k_{cat}/K_m for the mutant and good pancreatic juice stability, were MTX-3-*t*-butyl-Phe, MTX-3-cyclobutyl-Phe, MTX-3-cyclopentyl-Phe, MTX-3-cyclopentyl-Tyr, and MTX-3-cyclobutyl-Tyr. All were approximately 4000-fold or more stable in pancreatic juice than MTX-Phe, and all were hydrolyzed (at 25 °C) with second order rate constants of or greater than 10^5 M^{-1} s^{-1}.

Conjugation of Mutant hCPA1-T268G to an Antibody—To test the utility of the enzyme prodrug combination for ADEPT, hCPA1-T268G was conjugated to two antibodies. ING-1 binds to Epcam, a molecule expressed on a variety of epithelial cell tumors (46). Campath-1H binds to CDw52, which is expressed on a variety of T and B cells but not on most epithelial cells or tumors (30, 47). To couple hCPA1-T268G to these antibodies, a maleimide was placed on the enzyme with Sulfo-SMCC; this process had no effect on enzyme activity. A free thiol was then placed on the antibody with 2-iminothiolane. The hCPA1-T268G-antibody conjugate was then generated upon combina-

TABLE VIII
Enzyme activity and antigen binding affinity for conjugates

Conjugate	Specific activity[a]		Percentage bound to antigen[b]	K_d
	μmol/min/mg total protein	*μmol/min/mg hCPA1*[c]	%	*nM*
ING-1·hCPA1-T268G	4.2	22.2	85	0.4
C-1H·hCPA1-T268G	3.8	20	74	2.2

[a] Assayed with hippuryl-phenylalanine.
[b] For ING-1 conjugate, binding was performed on fresh, trypsininzed HT-29 cells. For C-1H conjugate, binding was performed with formaldehyde-fixed Wein-133 cells.
[c] Specific activity calculation assuming 1 mol of CPA/mol of antibody.

activity of conjugates toward hippuryl-Phe. Based upon the enzyme activity measured in this way, 1–1.5 mol of hCPA1-T268G appeared to be routinely associated with each mol of antibody. Table VIII also shows the percentage of the enzyme activity that bound to the relevant antigen along with the binding affinity as determined according to Lindmo (49). Both antibody conjugates expressed enzyme activity and bound to antigen. The percentage of conjugate that bound to antigen was 75–85%, and the K_d values are in agreement with literature indicating that the antigen combining region of the antibody was not adversely effected by the conjugation process

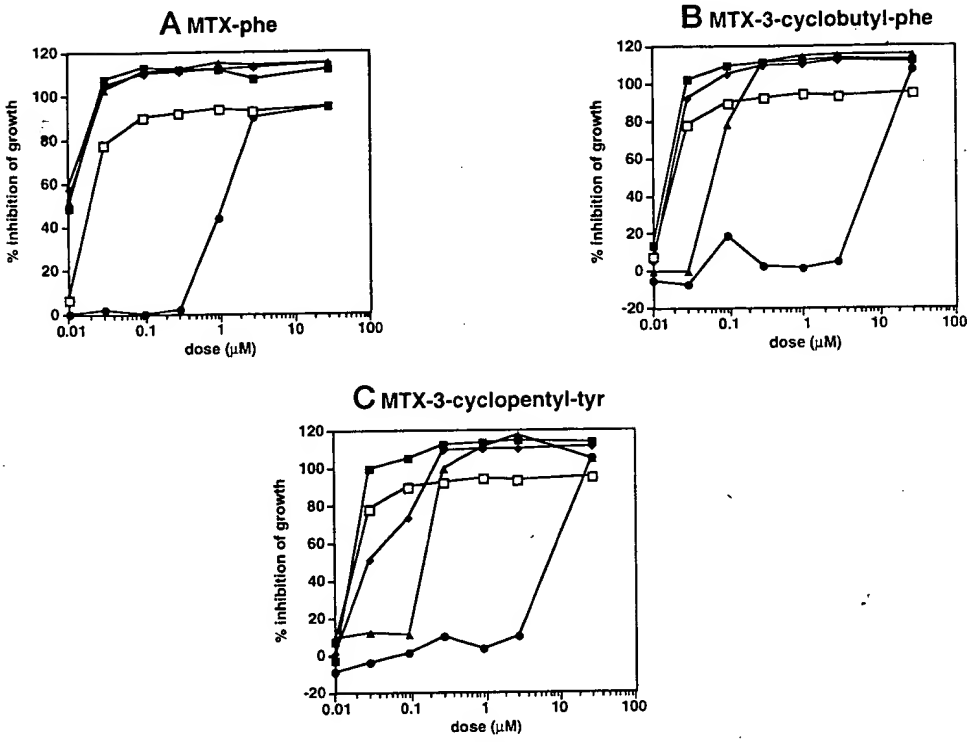


FIG. 3. **Use of the antibody-enzyme conjugate ING-1·hCPA1-T268G in a cell culture model of ADEPT.** HT-29 cells were grown in 95% RPMI 1640, 5% human serum. Then 50 μl of conjugate ING-1·hCPA1-T268G in fresh human growth medium was added to triplicate wells. Conjugate concentrations of 0 (*filled circles*), 2 (*filled triangles*), 10 (*filled diamonds*), and 50 (*filled squares*) μg/ml were used. After 1 h at 37 °C in a 5% CO_2 incubator, conjugate was removed, and the plates were washed three times with growth medium without conjugate. Finally, prodrug at 0, 0.01, 0.03, 0.1, 0.3, 1, 3, and 30 μM was added in 200 μl of fresh human growth medium, and cells were allowed to grow for 72 h. Prodrugs used were as follows. *A*, MTX-Phe; *B*, MTX-3-cyclobutyl-Phe; *C*, MTX-3-cyclopentyl-Tyr. In control experiments (*open squares*), MTX replaced conjugate plus prodrug.

component in fetal bovine serum was a competitive inhibitor of hCPA1 and hCPA1-T268G. This inhibitor greatly decreased the efficacy of both enzymes, resulting in the requirement for high concentrations of conjugate to produce an *in vitro* ADEPT response. Human serum also inhibited the enzymes, but the inhibition was less severe and most closely fit an uncompetitive kinetic model (data not shown). Since ADEPT was to be targeted to humans, the human serum conditions were considered the more relevant.

HT-29 cells, which were found to express Epcam but not significant amounts of CDw52, were seeded and allowed to adhere for 24 h. The cells on the plate were then incubated at 37 °C for 1 h with 0, 2, 10, or 50 μg/ml conjugate consisting of hCPA1-T268G coupled to either ING-1 or Campath-1H. Cells were then washed free of unbound antibody and allowed to grow for 72 h in the presence of varying concentrations of MTX-Phe, MTX-3-cyclobutyl-Phe, or MTX-3-cyclopentyl-Tyr. The results of these experiments are shown in Figs. 3 and 4 for the ING-1 and Campath-1H conjugates, respectively. The IC_{50} for MTX in these cells under these conditions was 10 nM. In the absence of conjugate, all three prodrugs were considerably less toxic than MTX. The stable prodrugs, MTX-3-cyclobutyl-Phe and MTX-3-cyclopentyl-Tyr, were 800 times less toxic than MTX (IC_{50} values for the two prodrugs were 8.5 and 7.8 μM, respectively). MTX-Phe was 200 times less toxic than MTX under these conditions.

When the cells were preincubated with the Epcam-specific conjugate ING-1·hCPA1-T268G, as expected all three prodrugs [illegible] Even the lowest dose of conjugate, 2

T268G mutant enzyme with these prodrugs shown in Table VII. At 10 and 50 μg/ml conjugate, the IC_{50} values for all three prodrugs approached that of MTX itself, indicating excellent conversion of all three prodrugs to the drug at these conjugate doses.

In contrast, when the cells were preincubated with the control conjugate Campath-1H·hCPA1-T268G, the IC_{50} values did not change with increasing conjugate. Thus, in the presence of 50 μg/ml Campath-1H·hCPA1-T268G conjugate, IC_{50} values for the three prodrugs were 2.5, 13, and 15 μM, respectively, not different from the prodrugs in the absence of conjugate. Therefore, activity of the ING-1·hCPA1-T268G conjugate was immunospecific. In summary, the data demonstrate that an immune specific conjugate of the hCPA mutant hCPA1-T268G, can convert *in vivo* stable MTX prodrugs to MTX in a cell culture ADEPT experiment.

DISCUSSION

ADEPT has the potential to greatly enhance the tumor selectivity of cancer chemotherapy by generating a means for tumor-specific synthesis of chemotherapeutic or other toxic agents. This is accomplished through staged, systemic delivery of components that localize to tumor sites and selectively generate active drug(s) at the tumor (2–7). System requirements for a successful ADEPT strategy include (*a*) a prodrug that is not activated to drug *in vivo* by host endogenous enzymes, (*b*) an enzyme capable of producing the drug from the prodrug at a rate sufficient to produce toxic levels of the drug at the specific site, and (*c*) an antibody capable of targeting and localizing the

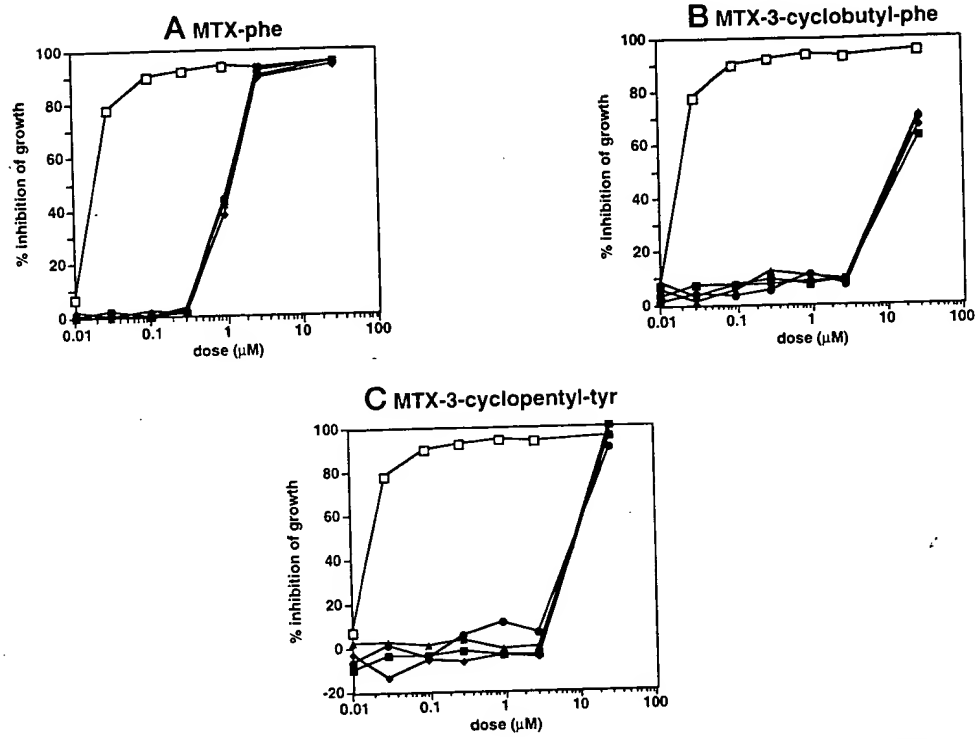


FIG. 4. **Use of the antibody-enzyme conjugate Campath-1H·hCPA1-T268G in a cell culture model of ADEPT.** Conditions and symbols were as in Fig. 3 except that Campath-1H·hCPA1-T268G replaced ING-1·hCPA1-T268G.

human and *E. coli* β-glucuronidase, bovine alkaline phosphatase, and others (2–29).

To permit repeat dosing with the ADEPT, an additional parameter is needed. A mechanism to decrease immune response to the ADEPT system is desirable. This goal is being approached by use of immune suppressing agents (5, 28) and, in one example, the use of a human enzyme (24). The latter system makes use of β-glucuronidase and takes advantage of the intralysosomal location of β-glucuronidase in mammalian cells by utilizing prodrugs with poor lysosomal access (24). Unfortunately, the enzyme is most active at acidic pH values not normal in the extracellular environment, decreasing the effectiveness of the extracellular targeted enzyme.

In an attempt to increase the repertoire of human enzymes available to ADEPT, we have investigated the potential utility of human CPA1 and CPA2. Heunnekens and colleagues (19–21) have suggested the use of bovine CPA along with amino acid prodrugs of MTX. Our initial experiments investigated the utility of this system directly. For this, we synthesized MTX-Phe and tested its substrate activity with the human CPA1 and CPA2. As reported for the bovine CPA, MTX-Phe is a good hCPA1 substrate and a fair hCPA2 substrate (21, 31). We also confirmed that the prodrug was stable in fetal bovine serum- and human serum-based growth media and was approximately 200 times less toxic than MTX in cell culture. However, MTX-Phe proved to be unstable *in vivo*, rapidly generating circulating and intestinal MTX. Indeed, even MTX-naphthyl-Ala, which is a poor hCPA1 substrate but is readily cleaved by

Design of Prodrugs That Are Not Hydrolyzed by WT hCPA and Are Hydrolyzed by Mutant hCPAs

Charged Prodrugs—Well known mammalian CPAs exist with substrate specificities for hydrophobic aromatic/aliphatic amino acids (those of the CPA class) and for basic amino acids (those of the carboxypeptidase B class). However, to our knowledge, mammalian carboxypeptidases that efficiently hydrolyze acidic C-terminal amino acids have not been reported. Thus, one of our first attempts to produce stable prodrugs was to produce natural and novel acidic amino acid α-peptides of MTX. Indeed, the prodrugs were considerably more stable in human pancreatic juice than MTX-Phe; MTX-2-carboxy-Phe and MTX-3-carboxy-Tyr, made for this purpose, were among the most stable prodrugs produced in our program.

To accommodate these negatively charged prodrugs in the hCPA hydrophobic binding pocket, Ile^{255} or Thr^{268} were mutated to Lys or His. However, we were unable to isolate these proteins from our yeast expression system. Presumably, the charged moiety in the hydrophobic environment prevented folding or active site alignment. Our inability to observe any protein from these mutants suggests that the three-dimensional structure was severely distorted.

One hCPA mutant did show some activity with the negatively charged aromatic prodrugs. The k_{cat}/K_m values for MTX-3-carboxy-Tyr and MTX-2-carboxy-Phe with hCPA1-T268G are 50- and 200-fold greater, respectively, than with WT hCPA. These two prodrugs are unique among the MTX prodrugs reported here in that they carry a negative charge *and* are bulkier than their respective Phe or Tyr parent. Thus, the rate

charged but are not bulky. Nonetheless, the absolute k_{cat}/K_m values for MTX-3-carboxy-Tyr and MTX-2-carboxy-Phe were each approximately 500 M^{-1} s^{-1}. Since this is 1000-fold poorer than WT hCPA1 with MTX-Phe, this enzyme/prodrug combination was not considered adequate for ADEPT, and improvements were sought.

Bulky Prodrugs—The width of the phenyl ring binding pocket in hCPA1 is minimally defined on one side of the ring by Thr^{268} (Fig. 2). The side chain for this amino acid is within 3.5 Å of the ortho- and meta-positions of the model inhibitor *N*-acetyl-Gly-Tyr. In hCPA2, this amino acid is Ala (31, 35), which according to our model of hCPA2 adds 0.5 Å to the width of the hCPA2 binding pocket compared with hCPA1 (although the change from Thr to Ala also increases peripheral space, which is not accounted for in this simple analysis). Consistent with a wider pocket, hCPA2 accepts bulkier aromatic substrates than hCPA1, as has been reported by others for Trp-based model substrates (37), as we have reported for MTX-naphthyl-Ala (31) and show in Table III for several compounds. To determine if this enlarged hCPA2 binding pocket compared with hCPA1 could be replicated in hCPA1, hCPA1-T268A was generated. We observed that the mutation increased the k_{cat}/K_m for hCPA1 approximately 500-fold for MTX-naphthyl-Ala to within a factor of 2 of WT hCPA2 with this compound, and a similar, although less dramatic, effect was also observed for MTX-3,5-diiodo-Tyr. Indeed, activity with hCPA1–T268A paralleled that of WT hCPA2 for most prodrugs made. The notable exception for this was MTX-Phe itself, which was used 250-fold more efficiently by hCPA1–T268A than WT hCPA2, suggesting that additional factors (such as amino acids 253 and 254) were also involved in substrate alignment.

To make use of the information that position 268 was important in defining the substrate pocket and to increase the pocket dimensions further, the size of the amino acid side chain at 268 in hCPA1 was decreased to "H" by mutating Thr to Gly. Based upon our model, this should increase the pocket width by 1.5 Å. This change only produced a modest, 2-fold, enhancement in the activity of the enzyme with MTX-naphthyl-Ala compared with hCPA1-T268A, suggesting that Ala at position 268 was sufficient for proper substrate binding and alignment of MTX-naphthyl-Ala. To explore this enlarged binding pocket further, the bulky amino acid prodrugs in Table I were designed to fit into the active site of our structural model for hCPA1-T268G. The compounds were synthesized and tested.

Determination of Optimal Binding Pocket Limits for Bulky Prodrugs

Tables III and VII show that the 2-iodo substituent on 2-iodo-MTX-Phe was accommodated by both WT hCPA1 and WT hCPA2. Indeed, k_{cat}/K_m of WT hCPA2 with MTX-2-iodo-Phe was 4-fold larger than with MTX-Phe. In the case of WT hCPA1, however, the k_{cat}/K_m for the iodo-substituted compound was approximately 4-fold smaller than that for MTX-Phe. This suggested that for hCPA1 the added size of the iodo substituent, which has a van der Waals radius of 4.23 Å along the C–I bond, was at the space limit of the binding pocket. The 1000-fold decrease in k_{cat}/K_m for the 2-cyclopentyl-substituted MTX-Phe, which has a van der Waals radius of 4.97 Å, measured along the bond connecting the substituent to the ring in the minimum energy cyclopentyl conformation, supports this notion. Incremental increases in the binding site for the 2-position MTX-Phe. The size of the 2-cyclopentyl substituent appears to be at the limit of the hCPA1-T268G binding pocket size, since k_{cat}/K_m for T268G with MTX-2-cyclohexyl-Phe (2-cyclohexyl with a minimum energy conformation van der Waals radius of 6.17 Å) was 200-fold lower than that for MTX-2-cyclopentyl-Phe. These data suggest that the T268G mutation in hCPA1 functionally increased the space at the ortho-position by at least 0.74 Å (4.97 − 4.23) to less than 1.94 Å (6.17 − 4.23). For comparison, calculations from our model give an added length in this dimension of 1.5 Å, in good agreement with the structure-activity relationship.

None of the meta-substituted prodrugs were WT hCPA1 substrates ($k_{cat}/K_m \leq 10$ M^{-1} s^{-1}) including the smaller compounds, 3-*t*-butyl- (van der Waals radius of 4.11 Å) and 3-cyclobutyl- (van der Waals radius of 4.5 Å). As in the case of the 2-substituted prodrugs, however, the hCPA1-T268G mutation exhibited activity with several of the bulky prodrugs with k_{cat}/K_m values similar to or better than WT hCPA1 with MTX-Phe. These included 3-*t*-butyl-, 3-cyclobutyl-, and 3-cyclopentyl-Phe and 3-cyclopentyl-Tyr. The 3-*n*-pentyl- (length of 7.11 Å) substituted prodrug appears to be beyond the limit of optimal substituent size for the meta-position. Since none of the 3-substituted compounds were active with the WT hCPA1, a functional measure of the hCPA1-T268 pocket increase at the meta-position binding site is not feasible, but calculations from our model yield a 1.5-Å increased length at this position as well.

The activities of WT hCPA2 with the 3-substituted compounds were all low, but detectable activity with the 3-cyclobutyl-, 3-*t*-butyl-, and 3-cyclopentyl-Phe, which were inactive with WT hCPA1, suggests the presence of more space in the meta-position binding site in hCPA2 than in the corresponding site in hCPA1, as expected. As with the 2-substituted prodrugs, the activity of hCPA1-T268A mimicked that of WT hCPA2, supporting the apparent similarities of the hCPA1-T268A and WT hCPA2 binding sites, although the correlation was not as tight as for the 2-substituted compounds.

Mutation of hCPA2 to A268G did not prove as effective as the T268G mutation for hCPA1. Thus, while the k_{cat}/K_m values for hCPA1-T268G with several of the 3-substituted prodrugs were over 100,000 M^{-1} s^{-1}, the k_{cat}/K_m for hCPA2-A268G with these compounds were all ≤10,000 M^{-1} s^{-1}. The prodrug that exhibited the largest k_{cat}/K_m enhancement with this enzyme was MTX-3,5-diiodo-Tyr (k_{cat}/K_m for the mutant = 520,000 M^{-1} s^{-1} compared with k_{cat}/K_m for the WT hCPA2 = 610). However, this compound was hydrolyzed too rapidly by pancreatic juice and as such was not useful. The compound may suggest, however, that other 3,5-disubstituted prodrugs should be explored for hCPA2-A268G.

Summary Analysis of Optimal Enzyme/Prodrug Combination

The 3-cyclopentyl substituent on either Tyr- or Phe-based prodrugs produced very poor enzyme activity with both hCPA1 and hCPA2. This poor enzyme activity resulted in excellent stability of the 3-cyclopentyl-substituted prodrugs in pancreatic juice. The hCPA1-T268G mutation returned the enzyme activity of both the Tyr- and Phe-based 3-cyclopentyl prodrugs to produce k_{cat}/K_m values approaching that of WT hCPA1 with MTX-Phe. The similar mutation on hCPA2 was not effective. Therefore, the optimal enzyme/prodrug combination from the current analysis is hCPA1-T268G with either Tyr- or Phe based 3-cyclopentyl prodrugs.

that cannot be accounted for in our model. Thus, the k_{cat}/K_m of hCPA1-T268G with the 3-cyclobutyl prodrug was at least 100,000 times greater than that of WT hCPA1 with this prodrug and actually 4 times better than WT hCPA1 with MTX-Phe. In contrast, mutation of hCPA2 to hCPA2-A268G enhanced the activity with this compound less than 10 times, and the absolute k_{cat}/K_m for the mutant was about 40 times poorer than WT hCPA2 with MTX-Phe. Similarly, the 3-cyclopentyl-Phe, 3-*n*-pentyl-Phe, 3-*t*-butyl-Phe, 3-cyclopentyl-Tyr, 3-cyclobutyl-Tyr, and 3-*t*-butyl-Tyr prodrugs were all 10–1000-fold better substrates for hCPA1-T268G than for hCPA2-A268G. These differences define some as yet undetermined deficiency in our model and stress the importance of result-directed model refinement, as used here, for this type of enzyme and prodrug design process.

Cell Culture ADEPT

For the purposes of ADEPT, the kinetic data indicate that hCPA1-T268G is the best suited enzyme. This conclusion is based on the excellent stability of 3-substituted Phe and Tyr prodrugs in pancreatic juice and their equally excellent hydrolytic rates with the hCPA1-T268G. This conclusion led us to test the combinations further in a series of *in vitro* ADEPT experiments. In these experiments we showed that only antigen-specific binding of conjugate to tumor cells (*i.e.* the ING-1·hCPA1-T268G conjugate with HT-29 cells) was able to generate an ADEPT response. Importantly, the data indicate that the tumor cell-bound enzyme is capable of generating MTX from otherwise *in vivo* stable prodrugs at relevant conjugate and prodrug concentrations. Indeed, in the presence of sufficient conjugate, the IC_{50} of the prodrugs tested approached that of MTX itself. Since MTX efficacy is dependent upon both the concentration and time of exposure, the IC_{50} data indicate that MTX was rapidly and quantitatively generated in these cases.

The relative efficiency at which ING-1·hCPA1-T268G used the prodrugs tested in the cell culture experiments can be assessed by comparing the amount of conjugate required to drop the prodrug IC_{50} to that of MTX itself. For MTX-3-cyclopentyl-Tyr, the amount of conjugate needed was 10 μg/ml. In comparison, for MTX-Phe the amount needed was less than 2 μg/ml, since at all conjugate doses tested, MTX-Phe was as active as MTX itself. This is consistent with the relative k_{cat}/K_m values for these compounds with hCPA1-T268G (0.16 *versus* 7.35×10^6 M^{-1} s^{-1} for MTX-3-cyclopentyl-Tyr and MTX-Phe, respectively).

The conjugate doses of 2–10 μg/ml required here can be compared with those reported for other enzyme prodrug systems. Thus, others have reported the requirement of 0.25–10 μg/ml for antibody-enzyme conjugates using β-lactamases (12, 13), 1–10 μg/ml for *E. coli* β-glucuronidase (22, 23), 10–50 μg/ml for bovine alkaline phosphatase, and 10–100 μg/ml for penicillin-G amidase (8, 9). This comparison suggests the efficiency of the current system is comparable with that of most other systems.

The β-lactamase system with a doxorubicin prodrug appears to be more efficient than that reported here. However, the selectivity of the current system appears to be greater than that of the β-lactamase/doxorubicin system, since in the absence of enzyme, the doxorubicin prodrug was only 7-fold less toxic than doxorubicin itself, while the MTX prodrugs were 800-fold less toxic than MTX (13). Nonetheless, the greater efficiency of the β-lactamase/doxorubicin system suggests that serum was the more severe, inhibition of these enzymes by human serum was significant. Further site-directed or random mutagenesis experiments may permit these refinements. The important advantage of the current system in using a human enzyme warrants this additional refinement.

In summary, we have generated a novel human enzyme/prodrug combination for MTX (and potentially other antifolates) prodrugs. This system was realized through structure-driven site-directed mutagenesis of hCPA1 in parallel with structure-driven design of *in vivo* stable prodrugs of MTX. The work provides the first steps for the concept of generating *in vivo* stable prodrugs of MTX and a human enzyme capable of hydrolyzing them. The utility of this concept may extend well beyond the current enzyme prodrug system and provide a general methodology to make use of the wealth of structural data now available on a variety of human enzymes to design other *in vivo* stable prodrugs and mutate these enzymes to accept new prodrugs. The advantage that the current system may have for clinical use is the likely low immunogenicity of the mutant hCPA1 along with the known toxicity profile of MTX.

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